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*Advances in Brief*

# E1A Inhibition of Radiation-induced NF-κB Activity through Suppression of IKK Activity and IκB Degradation, Independent of Akt Activation[1]

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## Abstract

**Activation of the transcription factor nuclear factor κB (NF-κB) has been implicated in the protection of cells from apoptosis. We have shown previously that the adenovirus type 5 E1A sensitizes cells to radiation-induced apoptosis by inhibiting NF-κB activity. However, the exact mechanism of inhibition is not known. In this study, we compared the activity of inhibitor of nuclear factor-κB (IκB) kinase (IKK) and the degradation of IκBα in E1A transfectants and parental human cancer cells after ionizing radiation treatment. We found that radiation-induced IKK activity and IκBα degradation were inhibited in the E1A transfectants. Recently, Akt has been implicated in NF-κB activation. To test whether Akt is regulated by E1A and is involved in radiation-induced NF-κB activity, we examined the phosphorylation status of Akt in the E1A transfectants and parental cells and in irradiated cells. The results indicated that radiation induced Akt phosphorylation and that E1A inhibited basal but not radiation-induced Akt phosphorylation. We additionally examined radiation-induced NF-κB activity in cells stably transfected with a dominant-negative, inactive Akt and in parental cancer cells treated with a phosphatidylinositol 3-kinase inhibitor, wortmannin. We found that dominant-negative Akt and wortmannin did not block radiation-induced NF-κB activity. Thus, our results suggest that inhibition of IKK activity and IκB degradation is the predominant mechanism for E1A-mediated inhibition of radiation-induced NF-κB activity and that radiation-induced Akt activation cannot be inhibited by E1A and is likely independent of radiation-induced NF-κB activity.**

## Introduction

IR[3] therapy is one of the most common cancer treatments. However, the therapeutic efficacy of this method decreases when cancer cells develop resistance to radiation. Ad5 E1A expression has been shown to sensitize cells to IR, anticancer agents, TNF-induced apoptosis (1–6). Ad5 E1A was also shown to function as a tumor suppressor, suppressing transformation, tumorigenicity, and metastasis (7–9). Induction of apoptosis may be one of the important functions of E1A tumor suppression (8–10). However, how E1A mediates inhibition of radiation-induced NF-κB activity is not clear.

NF-κB has been shown to play a critical role in blocking apoptosis induced by a variety of stimuli, including TNF, chemotherapeutic compounds, and γ-radiation (1, 6, 11). The most common form of NF-κB is a heterodimer of p65 and p50 subunits (12). In resting cells, NF-κB is sequestered in a latent form in the cytoplasm through association with IκBs, the endogenous inhibitors of NF-κB. In the usual pathway of NF-κB activation, IκBα is phosphorylated at Ser32 and Ser36 by the IKK complex, which consists of the IKKα, IKKβ, and IKKγ/NF-κB essential modulator subunits (12). The phosphorylated IκBα is additionally ubiquinated and degraded, resulting in NF-κB translocation into the nucleus to activate target genes. Some of these genes products, such as c-IAP1, c-IAP2, TRAF1, TRAF2, and Bf1/A1 have been shown to exhibit antiapoptotic functions (13, 14).

Recently, activation of the growth-factor-regulated serine/threonine protein kinase Akt (also known as protein kinase B) was shown to provide a survival signal that protects cells from apoptosis induced by various stresses (14). Activation of Akt was also shown to provide a critical cell survival signal required for tumor progression (15). Akt is downstream of PI3k. Activation of PI3k results in increase of the 3′-phosphorylated phosphatidylinositides P(3, 4, 5)P3 and P(3,4)P2. These phosphatidylinositides bind to the pleckstrin homology domain of Akt and induce translocation to the plasma membrane, where Akt is phosphorylated on Thr308 and Ser437 by phosphoinoside-dependent kinase 1 and phosphoinoside-dependent kinase 1/protein kinase C-related protein kinase-2 complex (16). The antiapoptotic effects of activated Akt include the phosphorylation of Bad, caspase 9, forkhead transcription factors, and IKKα (14, 17). The involvement of Akt activity in TNF-induced NF-κB activation is controversial (17, 18). It is not yet clear whether Akt might be involved in radiation-induced NF-κB activity.

In this study, we examined how E1A suppresses radiation-induced NF-κB activity, including IKK activity and IκBα degradation. In addition, we examined the effect of E1A on Akt phosphorylation and the effect of IR on Akt phosphorylation and its relationship with NF-κB activity.

## Materials and Methods

**Cell Lines and Culture.** The parental human ovarian carcinoma cell line SKOV3.ip1, ip1-E1A2, the Ad5 E1A transfectant of SKOV3.ip1, and ip1-Efs, a stable transfectant of SKOV3.ip1 cells carrying a 2-bp frameshift deletion in the E1A coding region, were established and cultured as described previously (1). dA17/i and dA31/i, which are dominant-negative mutant Akt (dn-Akt, K179M) transfectants of SKOV3.ip1, were established by cotransfection of the SKOV3.ip1 cells with a HA-tagged dn-Akt expression vector and pcDNA3. The culture conditions for the dn-Akt cells were the same as for the other cells, except that the medium was supplemented with 500 μg/ml G418. One week before the experiment, the G418-containing medium was replaced with the regular medium. Cells were irradiated with a $^{137}$Cs source emitting a fixed dose of irradiation (Irradiator model 0103; United States Nuclear Corporation).

**Immunoblotting.** Total cell lysates were subjected to 10% SDS-PAGE and blotted onto nitrocellulose membranes. Polyclonal antibodies against NF-κB (p65), IKKβ, and IKKα (Santa Cruz Biotechnology, Santa Cruz, CA), and Akt or p-Akt on Ser437 (Cell Signaling, Beverly, MA) were used.

**Immunocomplex Kinase Assay.** The immunocomplex kinase assay was performed as described previously (6). Briefly, ip1-E1A2, ip1-Efs and SKOV3.ip1 cells were treated with IR (5 Gy) or not irradiated. The cell lysates were incubated with anti-IKKβ antibody at 4°C overnight. The immunopre-

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[3] The abbreviations used are: IR, ionizing radiation; Ad5, adenovirus type 5; TNF, tumor necrosis factor; IκB, inhibitor of κB; IKK, IκB kinase; PI3k, phosphatidylinositol 3′-kinase; dn-Akt, dominant negative Akt; HA, hemagglutinin A; p-Akt, phosphorylated Akt.

cipitates were collected for the kinase assay with a glutathione *S*-transferase-IκBα substrate.

**Electrophoretic Mobility Shift Assay.** The electrophoretic mobility shift assays were performed as described previously (1). Briefly, the dn-Akt transfectants and SKOV3.ip1 cells were exposed to 5 Gy of IR, or SKOV3.ip1 cells were treated with 30 ng/ml of TNF (R & D Systems Inc., Minneapolis, MN) for 30 min. Nuclear extract (5 μg of protein) was incubated with 1 μg of poly(dI-dC; Pharmacia, Piscataway, NJ) on ice for 20 min, and a $^{32}$P-labeled double-stranded oligonucleotide containing the κB site from the human immunodeficiency virus was added. The probe was allowed to bind at room temperature for 20 min. The resulting complexes were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel. The mutant κB competitor was a double-stranded oligonucleotide containing mutations in the κB site (6).

## Results and Discussion

NF-κB activation requires degradation of IκBs to free NF-κB from the heterotrimeric IκB/NF-κB complex and to translocate it into the nucleus. IκBα is the major endogenous inhibitor of NF-κB activation. Because IR treatment has been shown to induce IKK activation and IκBα degradation, and E1A sensitizes cells to IR-induced apoptosis by inhibiting IR-induced NF-κB activity (1, 19), we asked whether suppression of IR-induced NF-κB by E1A may go through the IKK/IκBα pathway. To test whether E1A can inhibit IR-induced IκBα degradation, we examined the IκBα levels in parental SKOV3.ip1 cells, E1A transfectants, ip1-E1A2, and E1A frameshift mutant transfectants, ip1-Efs cells treated with IR. IR treatment reduced IκBα protein level in SKOV3.ip1 and ip1-Efs cells. However, in E1A-expressing ip1-E1A2 cells, IR did not change the IκBα protein level. Furthermore, the base level of IκBα is much higher in ip1-E1A2 cells. Similar results were also observed with the other sublines of E1A-transfected human ovarian cancer cells, SiE3 and SiE17 (data not shown). There was also more NF-κB protein in the ip1-E1A2 cells than in the control cells (Fig. 1*A*). This change is functionally insignificant, because NF-κB is known to be inactive in ip1-E1A2 cells (1). This could be attributable to stabilization of NF-κB by increased IκBα. We also used cycloheximide to block *de novo* protein synthesis in the cells during IR treatment. Under those conditions, we found that E1A prolonged the half life of IκBα (Fig. 1*B*). The graph in Fig. 1*B* shows the quantification of IκBα levels after irradiation. More than 80% of the IκBα protein in ip1-Efs and SKOV3.ip1 cells was degraded in 1.5 h after irradiation. However, <20% of the IκBα protein in the ip1-E1A2 E1A transfectant cells was degraded at the same time. The results indicated that E1A inhibited IR-induced IκBα degradation.

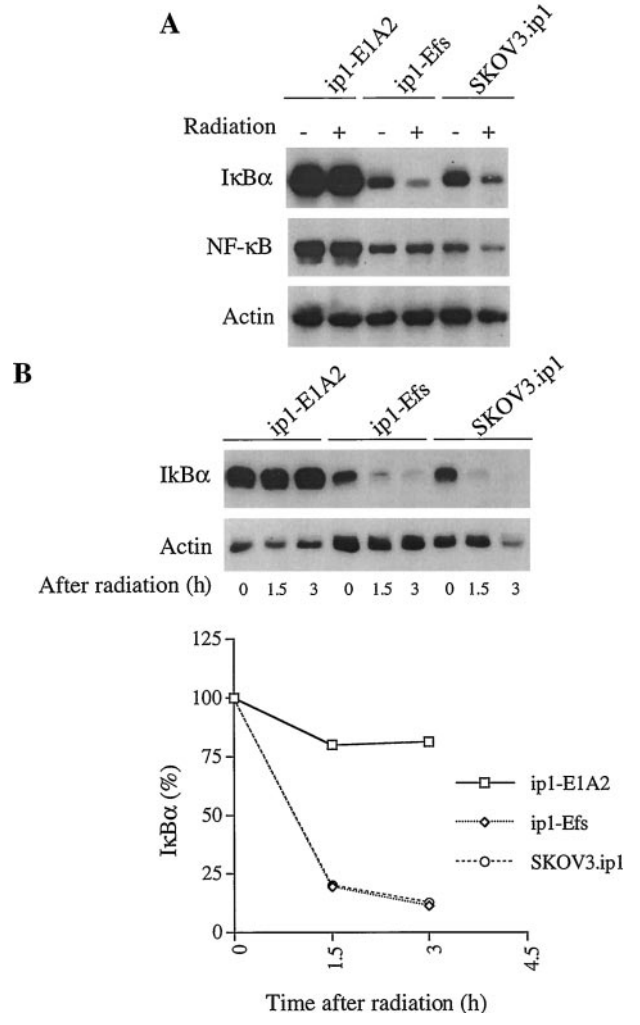


Fig. 1. Inhibition of IR-induced IκBα degradation mediated by E1A. ip1-E1A2, ip1-Efs, and SKOV3.ip1 cells were treated with 5 Gy of IR (+) or not treated (−). *A*, 2 h after irradiation, the cells were lysed. Equal amounts of total cell proteins were subjected to SDS-PAGE, and IκBα and NF-κB were detected by blotting with anti-IκBα and anti-NF-κB p65 antibodies. As a gel-loading control, the same blot was probed with an antiactin antibody. *B*, cells were cultured in cycloheximide immediately after irradiation, and cell lysates were prepared at the indicated times. Equal amounts of total cell proteins were subjected to SDS-PAGE for Western blotting. The graph shows IκBα protein normalized to actin protein and expressed as percentage of IκBα in an untreated sample measured with the NIH Image Program.

Whereas IKK activity has been shown to be necessary for IκB degradation and NF-κB activation induced by IR (19), X-ray-induced NF-κB activity was reported not to require IκBα degradation in rat astrocytes and human brain tumor cells (20). To evaluate the possible inhibitory mechanism of E1A on IκBα degradation, we measured the IKK protein levels and activity. Treatment of E1A-transfected and parental cells with IR had no effect on the protein level of IKKβ (Fig. 2*A*) and IKKα (data not shown). However, IR treatment increased IKKβ activity in E1A frameshift mutant transfected and parental cells but to a much lesser degree in E1A transfectants (Fig. 2*B*), indicating that E1A inhibited IR-induced IKKβ activity. The TNF-treated parental cells were used as a positive control.

Recent studies have shown that the involvement of Akt in the NF-κB pathway is a cell-specific activity (19). To test whether E1A mediates changes in Akt and, thus, is involved in IR-induced activation of NF-κB, we compared the phosphorylation of Akt in E1A transfectants and parental cells with or without IR treatment. The results indicated that E1A inhibited basal Akt phosphorylation (Fig. 3*A*). The data shown in Fig. 3*A* have been repeated a few times to confirm down-regulation of p-Akt by E1A (data not shown). Although the Akt level seems to be elevated slightly in the E1A transfectants shown in Fig. 3*A*, we did not observe the change in Akt protein level in other experiments. Thus, E1A inhibits phosphorylation of Akt but does not significantly affect Akt expression. Fig. 3*B* shows that IR induced Akt phosphorylation and that E1A did not inhibit IR-induced Akt phosphorylation. Because E1A inhibits IR-induced NF-κB activity (1) but not IR-induced Akt phosphorylation, it is conceivable that separate pathways may exist for IR-induced NF-κB and IR-induced Akt activation. To additionally investigate this phenomenon, we established cell lines stably transfected with dn-Akt and examined the IR-induced NF-κB DNA binding activity of those cells. The result indicates that IR-induced NF-κB activity was not blocked in the transfected cells (Fig. 4*A*), suggesting that Akt activation is not involved in IR induction of NF-κB activity. A similar result was observed with dn-Akt stable transfectants of the human breast cancer cell line MDA-MB-453 (data not shown). The bottom panel of Fig. 4*A* shows the expression of dn-Akt in the cell lines. To additionally confirm these observations, we treated the cells with the PI3k inhibitor wortmannin and IR. The results indicated that wortmannin could not

block IR-induced NF-κB DNA binding activity (data not shown). In addition to the DNA binding activity of NF-κB, we also confirmed that dn-Akt could not block IR-induced NF-κB activity as measured by a NF-κB-mediated promoter activity experiment (Fig. 4*B*). IR treatment increased luciferase activity in cells transfected with either dn-Akt plus κB-luciferase or empty vector plus κB-luciferase construct, and the IR-induced luciferase activity was not significantly different in dn-Akt-transfected cells and vector-transfected cells (Fig. 4*B*). Taken together, our results suggest that inhibition of IKK activity and IκBα degradation is involved in E1A-mediated inhibition of IR-induced NF-κB activity and that IR induces Akt phosphorylation, which is not required for IR-induced NF-κB activity.

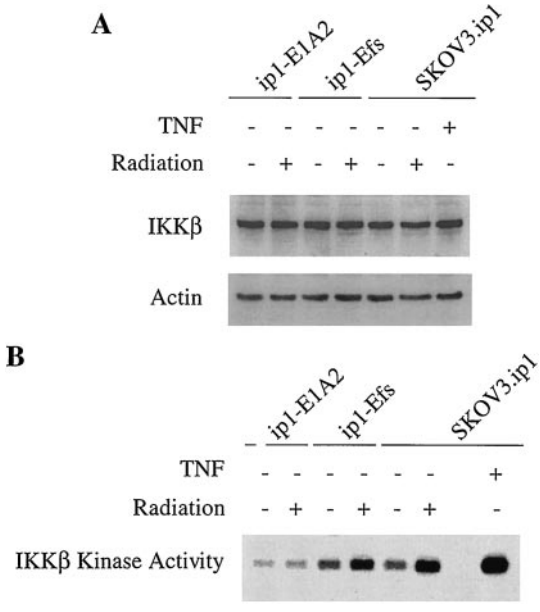


Fig. 2. E1A did not affect IKKβ protein level but inhibited IR-induced IKKβ activity. ip1-E1A2, ip1-Efs, and SKOV3.ip1 cells were exposed to 5 Gy of IR (+) or not treated (−). *A*, 2 h after irradiation, the cell lysates were prepared. Equal amounts of total cell proteins were subjected to SDS-PAGE, and IKKβ protein was detected by blotting with an anti-IKKβ antibody. As a gel loading control, the same blot was probed with an antiactin antibody. *B*, IKK complex was immunoprecipitated from the cell lysates with an anti-IKKβ antibody. IKK activity was measured with an immunocomplex kinase assay as described in "Materials and Methods." The TNF-induced IKKβ activity was used as a positive control. The IKKβ activity induced by IR was weaker as compared with that induced by TNF.

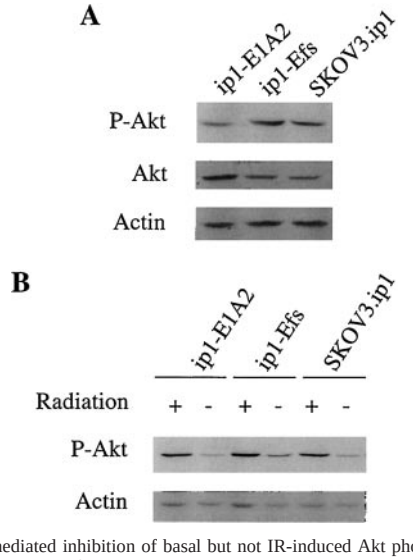


Fig. 3. E1A-mediated inhibition of basal but not IR-induced Akt phosphorylation. *A*, lysates of ip1-E1A2, ip1-Efs, and SKOV3.ip1 cells were subjected to immunoblotting. Anti-Akt and anti-p-Akt antibodies were used to detect Akt and p-Akt. An antiactin antibody was used to detect the actin protein as a loading control. *B*, ip1-E1A2, ip1-Efs, and SKOV3.ip1 cells were exposed to 5G y of IR (+) or not treated (−). After irradiation (2 h), cell lysates were prepared. Equal amounts of total cell proteins were subjected to SDS-PAGE, and p-Akt were detected.

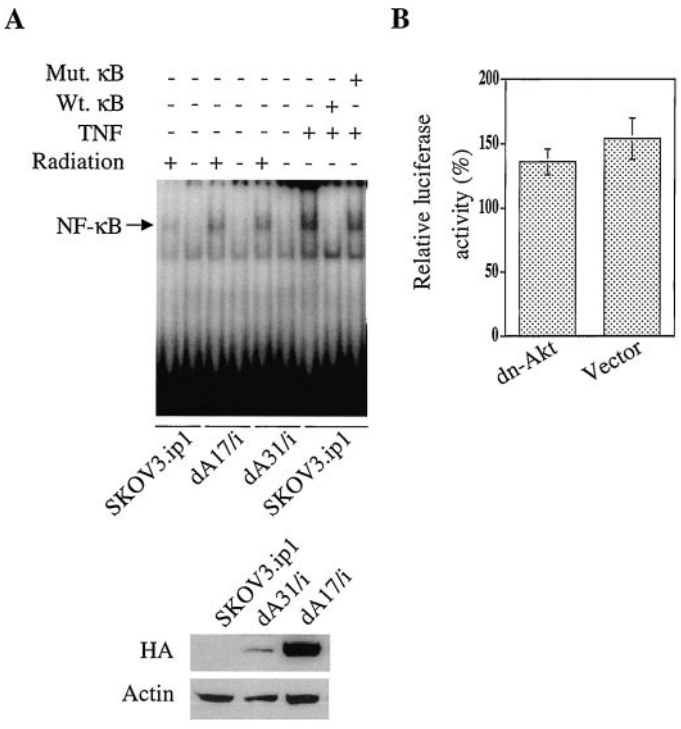


Fig. 4. dn-Akt did not block IR-induced NF-κB activity. *A*, HA-tagged dn-Akt stable transfectants (dA17/i and dA31/i) and SKOV3.ip1 cells were exposed to 5 Gy of IR (+) or not treated (−), or SKOV3.ip1 cells were treated with TNF (+). Nuclear extracts were prepared 2 h after exposure to IR or treatment with TNF for 30 min. The NF-κB DNA binding activity was measured by electrophoretic mobility shift analysis as described in "Materials and Methods." *Bottom panel*, expression levels of HA-tagged dn-Akt in the dA17/i and dA31/i cells as detected by Western blotting with an anti-HA antibody, and HA represented the dn-Akt level. *B*, SKOV3.ip1 cells were cotransfected with κB-luciferase, dn-Akt, or control vector and pRL-TK luciferase expression vectors. After transfection (48 h), the cells were exposed to 5 Gy of IR or not treated. Later (24 h), the cells were harvested, and luciferase activity was measured as described in "Materials and Methods." The κB-luciferase activity was normalized by the pRL-TK luciferase activity and calculated as a percentage of the luciferase activity of untreated cells. The data presented were the mean of three independent experiments; *error bars*, ± SD.

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